Human Keratinocyte-Lymphocyte Reactions In Vitro*

Brian J. Nickoloff, M.D., Ph.D., Teresa Y. Basham, Ph.D., Thomas C. Merigan, M.D., John W. Torseth, M.D., and Vera B. Morhenn, M.D.

Department of Dermatology (BJN, VBM) and Division of Infectious Disease, Department of Medicine (TYB, TCM, JWT), Stanford University School of Medicine, Stanford, California, U.S.A.

To extend our observation that recombinant γ interferon (r-IFN-γ) induces the synthesis and expression of HLA-DR antigen we have investigated 2 major areas including the modulation of r-IFN-γ-induced HLA-DR expression and the possible immunologic consequences of keratinocyte HLA-DR expression in vitro. The induction of keratinocyte HLA-DR expression was greater for continuous compared with pulse dosage (0.5–24 h) of r-IFN-γ and was markedly decreased after trypsinization of attached monolayers into single cell suspensions. The r-IFN-γ caused induction of HLA-DR and this was not influenced by either pretreatment with irradiation, PGE_2, or indomethacin. Both HLA-DR$^+$ and HLA-DR$^-$ cultured keratinocytes induced RNA synthesis and γ interferon production by allogeneic peripheral blood mononuclear leukocytes (PBMLs) indicating mononuclear cell activation. However, this activation was not followed by significant mitogenesis and only slightly increased levels of [^{3}H]thymidine incorporation (maximal = 5800 cpm) by the PBMLs was observed. Cultured keratinocytes apparently inhibit both lectin-driven and mixed-lymphocyte reactions by producing a soluble mediator which is not dialyzable, or inhibited by pretreatment with indomethacin or anti-α, -β, -γ interferon antibodies. These results suggest that lymphocyte-keratinocyte reactions in vitro are complex and may be mediated by a variety of cytokines, lymphokines, and prostaglandins.
J Invest Dermatol 87:11–18, 1986

The maintenance of cutaneous homeostasis probably depends on complex cellular interactions between keratinocytes (KCs), Langerhans cells (LCs), T lymphocytes, and other lymphoid cells (B lymphocytes, monocytes, natural killer cells, etc.).

Keratinocytes produce soluble factors which can stimulate lymphocyte proliferation [epidermal cell-derived thymocyte activating factor (ETAF)] as well as differentiation (thymopoietin) [1–3]. By contrast, human skin homogenates inhibit lymphocyte cytotoxicity and mitogen-induced lymphocyte transformation [4]. Defined KC-derived products which can inhibit lymphocyte proliferation include prostaglandin E_2 (PGE_2), and interferon (IFN) [5–7]. Moreover, lymphocytes, by producing γ interferon (IFN-γ), induce the synthesis and expression of HLA-DR by KCs, inhibit KC proliferation, and appear to influence KC differentiation in vitro [8–10]. Thus, KCs and mononuclear leukocytes may reciprocally influence each other's proliferation and differentiation via soluble factors such as cytokines, lymphokines, and prostaglandins.

Although we have demonstrated that KCs synthesize and express the light and heavy chains of the HLA-DR molecule after recombinant γ interferon (r-IFN-γ) exposure, the biologic significance of this HLA-DR expression by KCs is unknown [11]. The expression of HLA-DR on bone marrow-derived cells such as macrophages, T lymphocytes, and LCs is important in conferring antigen presentation capabilities, eliciting mixed lymphocyte reactions (MLRs) and allograft rejection [12]. Other investigators have studied the interaction between lymphocytes and other non-bone marrow-derived cells (e.g., endothelium, fibroblasts) both with and without IFN-γ exposure [13–16]. In this report we document our findings using KCs as the stimulator cell before and after r-IFN-γ exposure.

This paper covers 2 major areas. The first part includes studies of the modulation of IFN-γ induction of KC HLA-DR expression. The second part describes studies of the interaction between resting and activated, allogeneic, peripheral blood mononuclear leukocytes (PBMLs) using HLA-DR$^-$ and HLA-DR$^+$ cultured KCs.

MATERIALS AND METHODS

Preparation of Keratinocyte Cultures Single cell suspensions of normal skin obtained at the time of face-lift surgery were

Manuscript received August 6, 1985; accepted for publication February 7, 1986.

Supported by NIH 5R01AI05629-23 (TCM).

*Portions of this work were presented at the Western Regional Meeting and at the Joint International Meeting of The Society for Investigative Dermatology, Inc., and The Japanese Society for Investigative Dermatology, Washington, D.C., May 1–5, 1985 (J Invest Dermatol 84:302, 1985).

Reprint requests to: Brian J. Nickoloff, M.D., Ph.D., Director of Dermatopathology, R-166, Stanford University School of Medicine, Stanford, California 94305.

Abbreviations:
- BSA: bovine serum albumin
- Con A: concanavalin A
- DMEM: Dulbecco's modified Eagle's medium
- ELR: mixed epidermal cell-lymphocyte reaction
- ETAF: epidermal cell-derived thymocyte-activating factor
- FACS: fluorescence-activated cell sorter
- FCS: fetal calf serum
- FITC: fluorescein isothiocyanate
- HLA-DR: class II histocompatibility antigen
- IFN: interferon
- IL: interleukin
- KC: keratinocyte
- KLIF: keratinocyte-derived lymphocyte inhibitory factor
- LC: Langerhans cell
- MLR: mixed lymphocyte reaction
- PBML: peripheral blood mononuclear leukocytes
- PGE_2: prostaglandin E_2
- r-IFN-γ: recombinant gamma interferon

prepared according to the method of Liu and Karasek [17]. Small, round, viable cells were seeded on 3.5-cm collagen-coated Petri dishes (Lux, Flow Laboratories, Inc.) or 96-well flat-bottomed microtiter plates (Linbro, Flow Laboratories, McLean, Virginia) in Dulbecco's modified Eagle's medium (DMEM) containing 1.4 mM calcium, supplemented with 10% heat-inactivated fetal calf serum (FCS), 50 μg/ml of gentamicin, and 2 mM L-glutamine (complete medium). Seeding cell densities varied and are indicated for each experiment.

Viability as determined by trypan blue exclusion immediately following trypsinization was 90% or better. The cells were maintained in a humidified incubator with 5% CO_2/95% air at 37°C. In certain experiments, indomethacin (Sigma Chemical Co., St. Louis, Missouri) or PGE_2 (a gift of Dr. A. C. Allison, Syntex Corp., Palo Alto, California) was included in the complete media at the time of the initial seeding and in subsequent media changes. R-IFN-γ was added 2–3 days after cell seeding. All time points indicated refer to the time of addition of r-IFN-γ as day 0. Determination of cell number and viability utilizing a hemocytometer and Olympus inverted microscope was performed as previously described [10].

Interferon and Antibodies Against Interferons Human r-IFN-γ, generously supplied by Dr. G. Burton (Genentech Inc., South San Francisco, California), had a specific activity of 3.0×10^6 units/mg as determined by virus inhibition plaque assay.

Anti-α, -β, and -γ interferon antibodies were obtained from the National Institutes of Health. These reagents were sheep antisera (catalog nos. G-026-502-568, G-028-501-568; anti-α, and anti-β, respectively) and rabbit antisera (catalog no. G-034-501-565; anti-γ). Control antiseras (sheep and rabbit) were also utilized (catalog nos. G-027-501-568, G-035-501-565).

Measurement of γ Interferon γ-Interferon was measured in culture supernatants by a sensitive and specific radioimmunoassay (Centocor, Inc., Malvern, Pennsylvania) which employs a double-sandwich technique using one radiolabeled and a second fixed monoclonal antibody to biologically active human IFN-γ.

Keratinocyte HLA-DR Staining Keratinocyte HLA-DR staining involved trypsinization of cultured KC monolayers using 0.3% trypsin plus 1% EDTA. Aliquots of 10^6 viable cells were indirectly stained for 10 min on ice with 1 μg of monoclonal antibody (anti-HLA-DR; L243; Becton Dickinson Monoclonal Center, Mountain View, California) diluted in heat-inactivated FCS containing 0.1% sodium azide. Nonspecific background staining was determined with control isotypes. The cells were then washed and stained with 1 μg fluorescein isothiocyanate (FITC)-conjugated goat antimouse IgG (Becton-Dickinson). The fluorescence per cell was determined with a fluorescence-activated cell sorter (FACS III; Becton-Dickinson), and a histogram showing the number of cells against the intensity of fluorescence was recorded. All values presented have had nonspecific background and isotype control values subtracted.

Determination of PGE_2 Levels The radioimmunoassay for PGE_2 was performed using rabbit antihuman PGE-bovine serum albumin (PGE-BSA) serum (Miles-Yeda, Ltd.) according to the procedure described in the Miles bulletin. [^{3}H]Prostaglandin E_2 (New England Nuclear; sp act 210 Ci/mmol) was used as the tracer.

Preparation of Peripheral Blood Mononuclear Cells; Conditions of the Mixed Lymphocyte Reaction and Lectin Stimulation Peripheral blood mononuclear leukocytes, which were used as responder cells, were obtained by centrifugation of defibrinized venous blood over Ficoll-Hypaque density gradients. The interface cells were collected and washed with RPMI 1640 (Gibco, Grand Island, New York).

For control MLRs, allogeneic stimulator PBMLs were irradiated with 3000 rad from a Cs source and were combined with nonirradiated responder PBML in RPMI medium containing 10–15% recalcified human plasma (a gift of Dr. E. Engleman) to flat-bottomed 96-well culture plates. After addition of PBML, some of the 96-well plates were centrifuged at 200 *g* for 1–2 min. After 6 days of incubation in 37°C humidified air containing 10% CO_2, 10 μl of 0.1 mCi/ml [^{3}H]thymidine (sp act 6 Ci/mmol; Schwartz/Mann, Cambridge, Massachusetts) were added per well and the cultures harvested 18 h later on a PHD cell harvester (Cambridge Technology, Cambridge, Massachusetts). For the uridine incorporation studies, a final concentration of 5 μCi/ml [^{3}H]uridine (sp act 37.4 Ci/mmol; New England Nuclear, Boston, Massachusetts) was used during a 4-h pulse. Cell-associated radioactivity was measured by scintillation counting (Beckman Scintillation Counter, model LS 7000) and the results were expressed as the mean of triplicate cultures.

The results presented in Figs 4 and 5 represent the mean of 6 experiments and included the combination of $5–10 \times 10^4$ responder PBML, $5–10 \times 10^4$ irradiated allogeneic stimulator PBML, and various numbers of KC. Peripheral blood mononuclear cell size was determined by their light scatter on the FACS and by visualization in a hemocytometer. For dialyzed media, cell-free supernatants from attached cultured KC conditioned for various time intervals in RPMI media were obtained and dialyzed overnight at 4°C against 50 vol of RPMI (dialysis tubing molecular weight cutoff 3500, Spectrapor-Spectrum Medical Industries Inc., Los Angeles, California) and then sterilized by Millipore filtration. For mitogen stimulation, 5×10^4 PBML were incubated with concanavalin A (Con A, Pharmacia Fine Chemicals) and pulsed overnight with [^{3}H]thymidine (1 μCi/well) on day 3.

RESULTS

Modulation of r-IFN-γ Interferon Induced Keratinocyte HLA-DR Expression To extend our initial observations regarding r-IFN-γ induction of HLA-DR expression by cultured

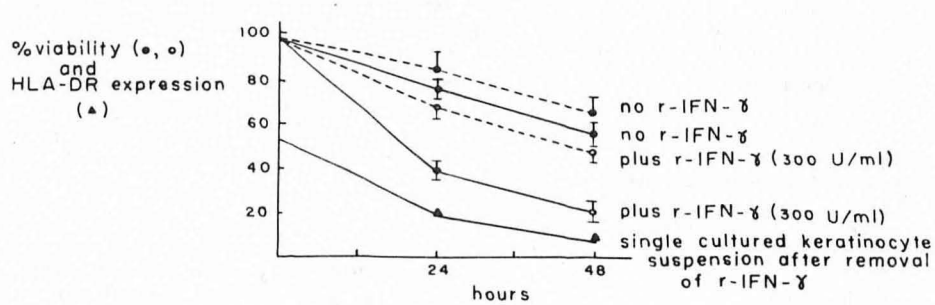

Figure 1. Viability and HLA-DR expression of trypsinized KC. *Solid lines:* Human KC cultured for 3 days in the presence or absence of r-IFN-γ (300 units/ml) trypsinized off collagen-coated Petri dishes and either stained with monoclonal antibody against HLA-DR immediately (t = 0) or maintained in RPMI medium. *Dashed lines:* Epidermal cells trypsinized from the skin. Immediately after trypsinization and washing, the cells were either treated with r-IFN-γ (300 units/ml) or untreated (controls). The percent viability is represented by *open* (plus r-IFN-γ) or *closed* (no r-IFN-γ) *circles*. HLA-DR expression on cultured KC trypsinized after r-IFN-γ treatment (3 days) (*triangles*).

KC we have exposed the KC to various conditions including: (1) trypsinization of HLA-DR expressing KC; (2) brief pulses rather than continuous exposure to r-IFN-γ; and (3) pretreatment of the KC with irradiation, PGE_2, indomethacin.

Viability and HLA-DR Expression of Trypsinized r-IFN-γ-Treated Keratinocytes: An initial comparison was made between the viability of trypsinized fresh epidermal cells and trypsinized cultured KC when both are maintained at 37°C in RPMI medium, both with and without r-IFN-γ (300 units/ml). Fig 1 reveals that the viability of trypsinized cultured KC is decreased compared with trypsinized fresh epidermal cells. The decline in viability is more marked after r-IFN-γ exposure for both cultured KC and fresh epidermal cells compared with untreated controls. The rapid decline in HLA-DR expression of the trypsinized cultured KC (Fig 1, triangles) parallels the decreased viability of the r-IFN-γ-treated cultured KC.

Comparison of Pulsing vs Continuous Exposure to r-IFN-γ on Cultured KC HLA-DR Expression: The degree of induction and disappearance of KC HLA-DR expression after a brief exposure of 0.5, 4, and 24 h to r-IFN-γ (500 units/ml) is different than with continuous exposure to r-IFN-γ (Fig 2). Less induction of HLA-DR expression occurs after pulsing with r-IFN-γ compared with continuous exposure on days 1–6 (not shown). Also, by day 6, no significant HLA-DR expression was detectable on the r-IFN-γ pulsed cultured KC. Cells previosuly pulsed with r-IFN-γ displayed decreased cell density and increased responsiveness for induction of HLA-DR compared with previously untreated controls on days 6, 8, and 10.

Influence of Irradiation, PGE_2, and Indomethacin Pretreatment of Cultured KC on HLA-DR Expression by r-IFN-γ: The modulation of the induction and disappearance of KC HLA-DR expression by pretreatment with irradiation (2000 rads), PGE_2 (0.1 μg/ml), or indomethacin (1 μg/ml) followed by continuous exposure to r-IFN-γ (300 units/ml) for 3 days with subsequent thorough washing is shown in Fig 3. Neither the induction of HLA-DR nor the disappearance of HLA-DR expression was significantly influenced by any of these pretreatment modalities. The rate of disappearance of HLA-DR expression after continuous exposure and then removal of r-IFN-γ is similar to our previous report [18]. Utilizing murine macrophages, it has been reported that PGE_2 (0.1 μg/ml; 3×10^{-6} M) modulates both induction and disappearance of Ia-like antigen expression after IFN-γ exposure [19].

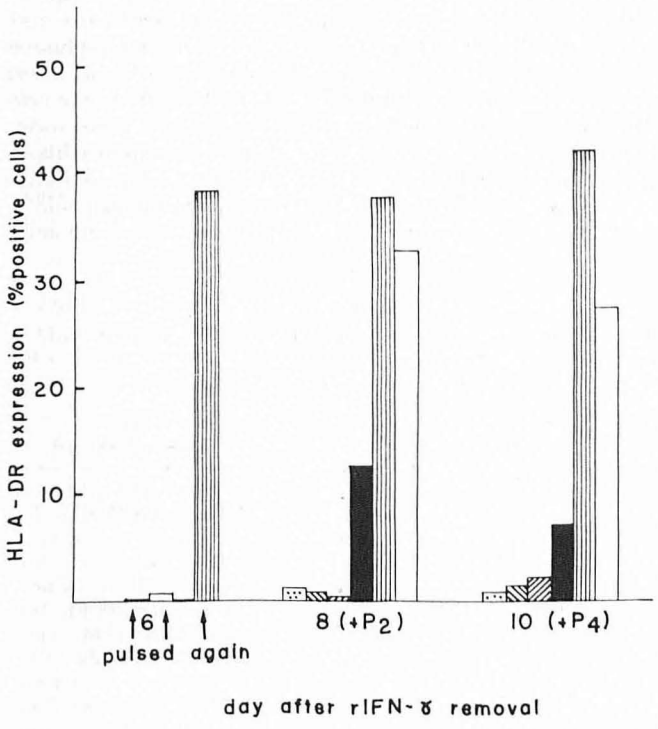

Figure 2. Comparison of pulsing vs continuous exposure to r-IFN-γ on cultured KC HLA-DR expression. Keratinocyte cultures were pulse treated with r-IFN-γ (500 units/ml), exposed continuously to r-IFN-γ (500 units/ml and 50 units/ml), or not treated. Six days later, the previously pulsed cultures as well as controls were again treated with a *continuous* exposure to r-IFN-γ (50 units/ml). □ = 30-min pulse, □ = 4-h pulse, ■ = 24-h pulse, □ = 500 units/ml r-IFN-γ continuously, □ = 50 units/ml continuously from day 0, □ = 50 units/ml continuously from day 6.

Resting and Stimulated Allogeneic PBML Interactions with HLA-DR Expressing Cultured Keratinocytes To determine the possible immunologic consequences of KC HLA-DR expression, we have examined a variety of conditions in which HLA-DR expressing KC are combined with allogeneic PBML.

Lack of [^{3}H]Thymidine Incorporation by Allogeneic PBML Cultured with Trypsinized Single Cell Suspensions of HLA-DR$^+$ Cultured KC: In the epidermal cell lymphocyte reaction (ELR) using epidermal cells (5×10^4) which had been trypsinized from skin immediately before use plus allogeneic responder PBML (5×10^4 cells), we consistently observed a stimulation index of 30–40, confirming our earlier observations [20]. However, despite a wide range in concentration of HLA-DR$^+$ cultured KC (10^3–10^5 cells) trypsinized to give single cell suspensions, no stimulation of allogeneic PBML was measurable by [^{3}H]thymidine uptake either on day 4 or day 7 after coincubation. Also, no stimulation of PBML occurred when we combined 5×10^4 trypsinized KCs with 25–75 $\times 10^3$ PBML. Because of the lack of stimulation by the single cell suspensions of cultured KC of allogeneic PBML and the poor viability and HLA-DR expression after trypsinization, we began to utilize KC which were grown and maintained attached on collagen-coated flat-bottomed microtiter wells in the presence of PBML.

Inhibiting Effect by Single Cell Suspensions of Cultured KC on MLR: To understand whether the lack of stimulation of allogeneic PBML by single cell suspensions of cultured DR$^+$ KC resulted from the presence of an inhibitor produced by the cultured KC or from poor viability and HLA-DR expression during the initial 48 h of incubation with PBML, we added various concentrations of trypsinized, cultured KC into a conventional MLR containing 5×10^4 responder PBML and 5×10^4 irradiated stimulator allogeneic PBML. Trypsinized single cell suspensions of cultured KC inhibited the MLR in a concentration-dependent

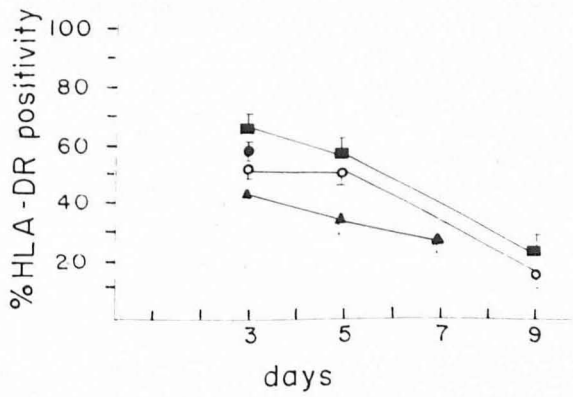

Figure 3. Modulation of induction and disappearance of KC HLA-DR expression by PGE_2, indomethacin, irradiation, and removal of r-IFN-γ. On day 0, the cells were treated by exposing them to r-IFN-γ (300 units/ml) plus indomethacin (1 μg/ml, *solid triangles*), PGE_2 (0.1 μg/ml, *open circles*), irradiation (2000 rads, *solid circles*) compared with control, untreated cells (*solid squares*). On day 3, the r-IFN-γ was removed, the cultures washed with complete media, and the cells were stained for HLA-DR on days 3–9.

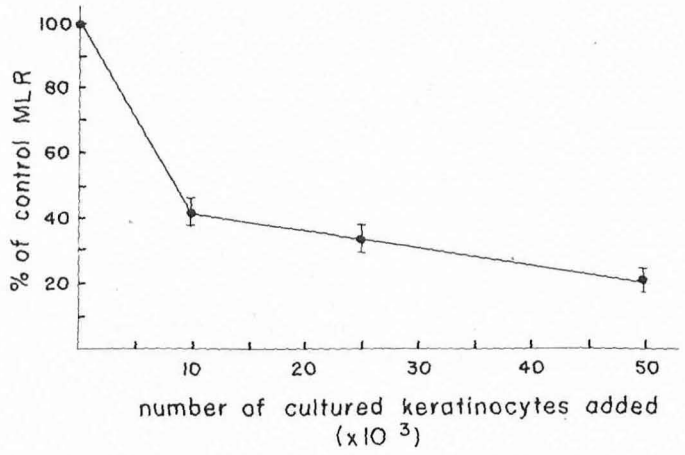

Figure 4. Inhibition of MLR by single cell suspensions of trypsinized, cultured KC. PBML (5×10^4) from responder A were incubated with 5×10^4 PMBL from responder B in the presence or absence of the indicated numbers of cultured, trypsinized KC. The average [^{3}H]thymidine incorporation for the control MLR (without KC) was 21,686 ± 1,259.

fashion (Fig 4). No significant difference in the inhibition was seen utilizing either r-IFN-γ pretreated and washed (100–300 units/ml) or untreated KC or whether we analyzed the MLR on day 4 rather than day 7. Preincubating the cultured KC with indomethacin (1 μg/ml) or anti-α, -β, or -γ interferon antibodies (10^2–10^3 units/ml neutralizing activity) did not prevent this inhibition.

Interaction of Attached, Cultured KCs, and Allogeneic PBML: When KC were seeded at 1–2 × 10^4 cells/plate and treated with r-IFN-γ for 3 days to induce HLA-DR expression prior to the addition of allogeneic PBML, the r-IFN-γ-treated KC sometimes resulted in a slight stimulation (Table I) of PBML [^{3}H]thymidine incorporation (range 947–5822 cpm) and this was greater than with the untreated KC (range 264–1173 cpm). In 6 experiments (boldfaced in Table I), the PBML [^{3}H]thymidine incorporation was significantly greater ($p < 0.05$) for the r-IFN-γ-treated KC compared with the untreated KC. These 6 experiments have the relative stimulation ratios (r-IFN-γ-treated vs untreated) provided in parentheses.

Cultured, attached KC at higher KC seeding densities (5–10 × 10^4 cells/plate) did not stimulate allogeneic PBML either with or without r-IFN-γ treatment or irradiation. No significant stimulation of PBML was observed when the attached, cultured KC (either with or without r-IFN-γ treatment) were preincubated with indomethacin (1 μg/ml), during incubation with allogeneic PBML.

Inhibitory Effect by Attached Cultured KC on MLRs: To explore the possibility that attached cultured KC produce a product that inhibits lymphocyte proliferation, we performed a conventional MLR on top of attached, cultured KC. At 5–10 × 10^4 seeded KC/microtiter well a concentration-dependent inhibition of the MLR occurred (Fig 5). By contrast when 2 × 10^4 KC/well were preirradiated with 2000 rads before incubation with 2 different sets of allogeneic lymphocyte populations, only a 4% inhibition of the conventional MLR resulted. This slight inhibition was abolished totally when the preirradiated KC cultures were also pretreated with indomethacin (1 μg/ml) suggesting that irradiated KC seeded at low density could no longer produce a factor in sufficient quantity to inhibit lymphocyte proliferation. The inhibitory effect of the attached, cultured KC at higher seeding densities could not be reversed by pretreatment with either 2000 rad, indomethacin, or anti-α, -β, or -γ interferon antibodies (5 × 10^2–10^4 units/ml neutralizing activity). To establish whether the allogeneic PBML may be killing the cultured KC, we determined the number of attached KC per microtiter well before and after incubation with allogeneic PBML. The addition of 5 × 10^4 allogeneic PBML did not change the number of viable KC per well after 48 h of coincubation (data not shown). Moreover, the number and viability of the allogeneic PBML also did not change at 48 h and at 6 days of incubation on either r-IFN-γ-treated or untreated KC monolayers (data not shown).

Keratinocyte Monolayer-Conditioned RPMI Media Inhibits Lymphocyte Proliferation: RPMI media conditioned from 24–96 h on attached cultured KC was removed and compared with fresh unconditioned RPMI medium for its ability to inhibit lymphocyte proliferation (Table II). The degree of inhibition of the MLR was dependent on the length of time the RPMI media had been conditioned on attached cultured KC. The change in pH of the media was also monitored and found to be slightly decreased with increasing time of conditioning by the cultured KC. The inhibition of lymphocyte proliferation by KC-conditioned media could be reversed by the addition of fresh complete medium (containing

Table I. [^{3}H]Thymidine Incorporation by Allogeneic Peripheral Blood Mononuclear Leukocytes (PBMLs) Incubated with Attached Cultured Keratinocytes[a]

Number of Keratinocytes Seeded	Treatment			
	None	2000 rad	r-IFN-γ[b]	r-IFN-γ + 2000 rad
10,000	480 ± 169	0	1594 ± 128	947 ± 169
	930 ± 493	1173 ± 387	**4232 ± 593 (2.3)**	**4680 ± 336 (2.3)**
20,000	264 ± 102	0	1673 ± 293	0
	929 ± 169	0	**5822 ± 628 (2.8)**	987 ± 227
		369 ± 89[c]		1424 ± 271
		711 ± 185		**3779 ± 1082 (4.0)**[c]
		756 ± 231		**3963 ± 628 (4.8)**[c]
		769 ± 202[c]		**4331 ± 370 (2.3)**[c]
50,000	0	0	1232 ± 618	0
	0	595 ± 229	1705 ± 805	491 ± 196
100,000	0	0	0	0
	0	0	480 ± 225	0

[a]The [^{3}H]thymidine incorporation represents the mean cpm of triplicate wells on day 7. Lymphocyte incorporation and keratinocyte (KC) incorporation alone (background) was subtracted. When the [^{3}H]thymidine incorporation by the PBML was statistically significant and increased for HLA-DR$^+$ over HLA-DR$^-$ KC, the ratio is provided in parentheses.

[b]Recombinant IFN-γ (300 units/ml) was added for 3 days, the cells were washed, and then allogeneic PBML were added. A replicate plate of KC was harvested at the time of PBML addition and HLA-DR expression by the KC was observed.

[c]The KC were preincubated with indomethacin (1 μg/ml).

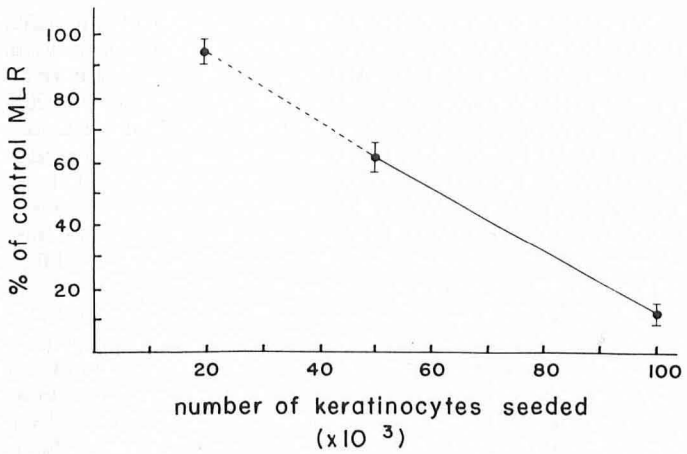

Figure 5. Inhibition of MLR by attached cultured KC.

10% human serum). However, the inhibition was not reversed following overnight dialysis.

To determine whether this inhibition could also be observed in another system, we examined the effects of conditioned KC medium on [^{3}H]thymidine incorporation by lectin stimulation of PBML. Keratinocyte medium conditioned for 48 h inhibited the Con A stimulation of PBML ($p < 0.05$). This inhibition of lymphocyte proliferation could not be reversed by preincubating the cultured KC (from which the conditioned medium was obtained) with either indomethacin (1 μg/ml) or anti-α, -β, or -γ interferon antibodies.

To evaluate the possible role of KC PGE_2 production, RPMI medium conditioned for 48 h was assayed for PGE_2 levels. After 48 h, cultured human KC produced nanomolar levels of PGE_2 (11.8 nM, Table III). Pretreatment with irradiation slightly reduced PGE_2 production (9.9 nM), whereas indomethacin (1 or 10 μg/ml) greatly reduced the PGE_2 levels (2.1 nM). By contrast, the addition of r-IFN-γ (500 units/ml) increased PGE_2 levels approximately 4.5-fold (54.2 nM). Similar trends were seen in a second, separate experiment.

Changes in PBML Cell Number and Cell Size Following Stimulation with Con A and/or DR$^+$ and DR$^-$ Cultured KC: Utilizing cultured endothelial cells and lectin stimulation of PBML, it has been previously suggested that [^{3}H]thymidine incorporation may not accurately reflect lymphocyte proliferation [21,22]. To determine whether this discordance could pose a problem in our system we analyzed Con A (50 μg/ml)-stimulated PBML on day 7. In keeping with previous reports, this lectin stimulation resulted in a 460% increase in cell number and a 50% increase in cell size. By contrast, when PBML were stimulated with Con A in the presence of 5×10^4 attached KC, only a 15% increase in cell number/plate occurred. (Some of this increase could have been due to detached KC.) However, on day 7, all Con A-treated PBML, whether incubated on KC monolayers or controls, had dramatically increased in cell size by 48%. This discrepancy between cell number and cell size in the experiments using lectin-stimulated PBML incubated on KC monolayers suggested that the KC may be producing factor(s) which selectively block lymphocyte mitogenesis but not activation.

To determine whether HLA-DR$^+$ KC when incubated with allogeneic PBML could cause a similar increase in the cell size, we assayed the cell size of PBML after incubation with r-IFN-γ-pretreated KC. No increase in cell size occurred using attached KC pretreated with 500 units/ml r-IFN-γ (results not shown).

Allogeneic PBML [^{3}H]Uridine Incorporation After Coincubation with Cultured KC With and Without Con A: It has been previously shown [23] that PGE_2 does not affect RNA synthesis of mitogen-activated lymphocytes and their entry into G_1, but does affect PBML mitogenesis (G_{1a}-G_{1b}). Therefore we assessed the effect of cultured KC on lymphocyte activation and RNA synthesis by measuring [^{3}H]uridine incorporation. On day 4, untreated (no r-IFN-γ exposure) cultured KC stimulated incorporation of [^{3}H]uridine by PBML approximately 3-fold (Table IV). The same stimulation also occurred when the cultured KC were pretreated with r-IFN-γ (500 units/ml). No detectable increase in PBML cell size or number either on day 4 or day 7 was seen despite the increased [^{3}H]uridine incorporation.

To add to our observations on the effect of cultured KC on [^{3}H]thymidine incorporation by lectin-stimulated PBML, we assayed [^{3}H]uridine incorporation in the absence and presence of cultured KC (Table IV). As expected, the [^{3}H]uridine incorporation in Con A-stimulated PBML was markedly increased over the PBML not stimulated with Con A. Furthermore, cultured KC had only a minimal inhibitory effect on [^{3}H]uridine incorporation by Con A-stimulated PBML as assessed on day 4.

Stimulation of IFN-γ Production by Allogeneic PBML and Cultured KC: Since either r-IFN-γ-treated or untreated cultured KC

Table II. Inhibitory Effect of Conditioned Media From Human Keratinocyte Monolayers on Mixed Lymphocyte Reactions (MLR) and Con A Stimulated Peripheral Blood Mononuclear Leukocytes (PBML)

Treatment	pH	[^{3}H]Thymidine Incorporation[a] (cpm)
Control MLR	7.15	18,392 ± 1,239
MLR in presence of 24-h conditioned medium[b]	6.9	12,063 ± 853[c]
MLR in presence of 96-h conditioned medium	6.8	6,906 ± 775[c]
MLR in presence of 24-h conditioned media diluted 1:2 with fresh complete medium		16,847 ± 1,002
Con A-stimulated PBML[d]		52,939 ± 1,849
Con A-stimulated PBML in 48-h conditioned medium		33,945 ± 1,628[c]

[a]The [^{3}H]thymidine incorporation is the mean of triplicate wells of 4 experiments in which background keratinocyte and lymphocyte [^{3}H]thymidine incorporation has been subtracted. Responder PBML (5×10^4) were incubated with 5×10^4 irradiated stimulator PBML in RPMI + 10% human serum.
[b]There was no reversal of this inhibition after overnight dialysis.
[c]This inhibitory response was statistically significant ($p < 0.05$).
[d]The concentration of Con A was 50 μg/ml.

Table III. PGE_2 Levels in 48-h Conditioned Keratinocyte (KC) Media

	PGE_2 Concentration (nM)
KCs	11.8
KCs + 2000 rads	9.9
KCs + indomethacin (1 μg/ml)	2.1
KCs + indomethacin (10 μg/ml)	2.6
KCs + r-IFN-γ (500 units/ml)	54.2

Table IV. [^{3}H]Uridine Incorporation of Peripheral Blood Mononuclear Leukocytes (PBML) Incubated with Keratinocytes and/or Con A

Treatment	[^{3}H]Uridine (cpm)
PBML alone	3,950
PBML + KC (± r-IFN-γ)	11,800
PBML + Con A (50 μg/ml)	210,650
PBML + Con A + KC	185,300

stimulate PBML to increase the [^{3}H]uridine incorporation (Table IV) and thus apparently do not block lymphocyte activation but do inhibit lymphocyte mitogenesis, we assayed the supernatants for IFN-γ production by allogeneic PBML coincubated with cultured KC. γ Interferon is known to be produced by activated T lymphocytes in response to alloantigens [23]. After 5 days of coincubation of allogeneic PBML with cultured KC, detectable levels of IFN-γ were present in the conditioned media (Table V). PBML alone produced no significant levels of IFN-γ and we confirmed the thoroughness of our washing techniques in these microtiter wells exposed to r-IFN-γ by assaying the supernatants from KC cultured alone. When allogeneic PBML were coincubated with DR$^+$ KC they produced more IFN-γ than with HLA-DR$^-$ KC, but an equivalent amount of IFN-γ (40–200 U/ml) was observed in subsequent experiments whether either HLA-DR$^+$ or HLA-DR$^-$ KC were used to stimulate the PBML.

DISCUSSION

Previous investigators have studied the modulation of induction of HLA-DR expression by IFN-γ [19,24]. We observed that unlike macrophages in KC, neither the r-IFN-γ induction nor disappearance of HLA-DR is influenced by pretreatment with irradiation, PGE_2, or indomethacin. Exposing the cultured KC to brief pulses (up to 24 h) of r-IFN-γ rather than continuous exposure produces a decreased percentage of KC expressing HLA-DR.

The interaction of allogeneic PBML with cultured KC is complex. To fully interpret the results it is important to note that T-cell stimulation first requires "activation" during which cells progress from G_0 to G_1 and then progression from G_1 through DNA synthesis and mitosis [25]. Our results indicate that attached cultured KC (either HLA-DR$^+$ or HLA-DR$^-$) can produce resting T-cell activation since these PBML secreted IFN-γ and showed increased RNA synthesis. However, the alloantigenic signal(s) by the KC did not lead to prominent mitogenesis ([^{3}H]thymidine incorporation). While the cultured KC when seeded at low cell density, and pretreated with r-IFN-γ to induce HLA-DR expression, did produce a small but statistically significant stimulation of [^{3}H]thymidine incorporation (maximal = 5800 cpm), the biologic importance of this result remains unclear. Clearly, HLA-DR$^+$, attached cultured KC are not as potent stimulators of allogeneic PBML mitogenesis as monocytes or LC [26] but appear to be similar to other non-bone marrow-derived HLA-DR$^+$ cells such as endothelial cells [13–16].

In a recent report an analogous approach was undertaken using HLA-DR$^+$ cultured fibroblasts and similar results regarding the lack of stimulation of resting allogeneic T lymphocytes were observed [27]. However, when IL-2 was added, purified T lymphocytes were stimulated to proliferate after coincubation with HLA-DR$^+$ fibroblasts. These authors concluded that HLA-DR$^+$ fibroblasts (unlike macrophages) do not stimulate adequate IL-2 production by allogeneic T lymphocytes to sustain lymphocyte mitogenesis.

Table V. γ Interferon Production by Allogeneic Peripheral Blood Mononuclear Leukocytes (PBML) After Coincubation with Cultured Keratinocytes

Cell Mixture[a]	γ Interferon (units/ml)[b]
PBML alone	0.1
r-IFN-γ treated KC alone (after 3 washes)	1.0
PBML + HLA-DR$^+$ KC	19.8
PBML + HLA-DR$^-$ KC	6.7
Control MLR	65.0

[a]PBML (10^5) were coincubated with 5×10^4 attached keratinocytes (KC). The HLA-DR$^+$ KC had been pretreated with 300 units/ml r-IFN-γ for 3 days prior to addition of PBML.

[b]The conditioned media was removed after 5 days of coincubation with PBML + KC and assayed using a radioimmunoassay.

Our results indicating that the presence of HLA-DR, in itself, on trypsinized KC is not sufficient to produce mitogenesis of allogeneic PBML may have several explanations. First, the single cell suspensions of HLA-DR$^+$ cultured KC have a poor viability compared with fresh epidermal cells and the HLA-DR$^+$ KC may not be viable in the RPMI media for a sufficiently long period to stimulate the allogeneic PBML. This explanation is less likely for the attached cultured KC as they maintained a much better viability and prolonged HLA-DR expression after removal of r-IFN-γ and exposure to allogeneic PBML. Second, the number of HLA-DR molecules expressed per cultured KC after r-IFN-γ in the dose range utilized (100–300 units/ml) may be lower than LC or monocyte HLA-DR cell surface expression and inadequate to produce optimal allogeneic PBML stimulation. Third, additional cell surface proteins besides HLA-DR may be important in allogeneic PBML reactions and these may not be inducible by r-IFN-γ treatment (such as HLA-DQ) in the KC [9]. Fourth, we did not type the specific histocompatibility antigens (class I or II) on either our stimulator or responder cells to document differences in their respective alleles. However, the lack of stimulation observed in many experiments could not be accounted for by a chance similarity in histocompatibility antigens between the different stimulator and responder cells. Fifth, the low level of stimulation of allogeneic PBML by cultured HLA-DR$^+$ KC may be due to the presence of inhibitory factors produced by the cultured KC as has been previously suggested [4,26]. To investigate the latter alternative we measured PGE_2 levels and have shown them present in nanomolar amounts in the medium and to be increased after r-IFN-γ exposure.

Attached cultured KC produced significant amounts (11.8 nM) of PGE_2. This production was slightly decreased by irradiation and markedly decreased with indomethacin pretreatment. Many previous investigators have observed that nanomolar concentrations of PGE_2 inhibit the [^{3}H]thymidine incorporation by lymphocytes after alloantigen reactions or lectin stimulation [28–32]. With respect to the mixed lymphocyte reaction, it has been reported that PGE_2 levels present in the culture supernatants range from 10^{-8}–10^{-9} M [32]. While 1.7 nM PGE_2 decreased the MLR and [^{3}H]thymidine incorporation by 38%, whole macrophage supernatants that contained identical PGE_2 levels inhibited the MLR by >70%, suggesting that additional factors besides PGE_2 may be responsible for inhibition of the MLR. In our system the attached cultured KC produced a 40–80% inhibition of the MLR depending on the number of KC seeded. We did not observe any striking effect on this inhibition with the use of indomethacin. This result suggests that PGE_2 production, while it may have contributed to some of the observed inhibition, does not account for all of it, and additional inhibitory factors also may be secreted. The presence of an inhibitory factor(s) (larger than 3500 daltons) could also be inferred from the dialysis experiment in which overnight dialysis did not alter the observed inhibition. However the role of PGE_2 in our experimental system is complex, since IFN-γ (which we added exogenously, and which is present in activated PBML supernatants [7]) greatly increased the KC production of PGE_2 (approximately 4-fold to 54 nM). Other investigators have observed that human IFN can increase the production of PGE_2 by nonimmune cells such as cultured fibroblasts [33,34].

During lymphocyte–keratinocyte interactions, variable levels of PGE_2 may be produced, and further studies with serial addition of multiple inhibitors of PG synthesis (indomethacin plus meclofenamate plus ETYA) with reduction of PGE_2 levels below 1 nM will permit more detailed analysis of additional soluble KC-derived inhibitors of PBML mitogenesis.

Because of the lack of effect of dialysis and the pretreatment with indomethacin, as well as the increase in [^{3}H]uridine incorporation in PBML cultured on KC monolayers, our data suggest the existence of an additional KC-derived lymphocyte inhibitory factor (which we have abbreviated KLIF). KLIF is probably different from the epidermal cell-derived inhibitor reported by

Tsuchida et al [35], as their inhibitor of a cytotoxic T-lymphocytic reaction could be reversed by preincubating the epidermal cells with indomethacin (0.1–1.0 μg/ml) and was tentatively identified as PGE_2. KLIF is probably different than the inhibitory factor identified by Sauder et al [1] as it is nondialyzable. KLIF may be similar, if not identical, to the recently described "epidermal cell derived lymphocyte differentiation factor" [36]. KLIF probably has a similar effect on PBML as does PGE_2 as it does not inhibit the "activation" signal for lymphocyte transformation, but does influence [^{3}H]thymidine incorporation by PBML as well as lymphocyte mitosis.

Since PGE_2 inhibits IL-2 production, the interaction of PBML and KC may be influenced by the addition of exogenous recombinant IL-2 [28]. It is possible that with addition of IL-2, the allogeneic resting T lymphocytes will be able to progress from activation to mitogenesis when coincubated with cultured HLA-DR^+ KC in a similar fashion as with HLA-DR^+ fibroblasts [27]. Since cultured KC stimulate PBML [^{3}H]uridine incorporation and IFN-γ production, it will be interesting to establish which other proteins result during this period of increased RNA synthesis. If terminal deoxynucleotidyl transferase [4] is one of these proteins, then KLIF may actually be capable of influencing PBML mitogenesis and differentiation [25].

It is important to further purify the KC-derived inhibitors of lymphocyte proliferation because these substances may be as important as lymphocyte stimulating factors such as ETAF and thymopoeitin in cutaneous homeostasis. Also, until these soluble inhibitors can be modulated or suppressed, further investigations into the biologic role of KC HLA-DR expression and keratinocyte–lymphocyte interactions will be hampered.

Our results underscore the complexity of resting T-lymphocyte activation and proliferation. PBML appears to recognize KC alloantigen and respond with IFN-γ secretion suggesting that this in vitro system may permit further dissection of the role of IFN-γ in lymphocyte–keratinocyte interactions.

Note: Since acceptance of this manuscript, Hayashi and Aurelian have isolated nonprostaglandin soluble factor(s) derived from murine UVB-irradiated epidermal cells coincubated with HSV-2 antigen which suppresses T-lymphocyte proliferation in vitro (J Immunol 136:1087–1092, 1986).

The authors thank Eva A. Pfendt who provided excellent technical assistance, Ruei-Chen Chin for performing the PGE_2 assays, and Dr. A. C. Allison for reviewing the manuscript.

REFERENCES

1. Sauder DN, Carter C, Katz SI, Oppenhiem JJ: Epidermal cell derived thymocyte activating factor. J Invest Dermatol 79:34–39, 1982
2. Chu AC, Patterson JAK, Goldstein G, Berger CL, Takezaki S, Edelson RL: Thymopoietin-like substance in human skin. J Invest Dermatol 81:194–197, 1983
3. Rubenfeld MR, Silverstone AE, Knowles DM, Halper JP, DeSostoa A, Fenoglio CM, Edelson RL: Induction of lymphocyte differentiation by epidermal cultures. J Invest Dermatol 77:221–224, 1981
4. McKinney C, Rzepiela AJ, Penneys NS: Inhibition of lymphocyte cytotoxicity and mitogen-induced transformation of lymphocytes by human skin homogenates. J Invest Dermatol 72:153–156, 1979
5. Jonnson CE, Anggard E: Biosynthesis and metabolism of prostaglandin E_2 in human skin. Scand J Clin Lab Invest 29:289–296, 1972
6. Schnipper LE, Levin M, Crumpacker CS, Gilchrest BA: Virus replication and induction of interferon in human epidermal keratinocytes following infection with herpes simplex virus. J Invest Dermatol 82:94–96, 1984
7. Shalaby MR, Weck PK, Rinderknecht E, Harkins RN, Frane JW, Ross MJ: Effects of bacteria-produced human alpha, beta, and gamma interferons on in-vitro immune functions. Cell Immunol 84:380–392, 1984
8. Basham TY, Nickoloff BJ, Merigan TC, Morhenn VB: Recombinant gamma interferon induces HLA-DR expression on cultured human keratinocytes. J Invest Dermatol 83:88–92, 1984
9. Basham TY, Nickoloff BJ, Merigan TC, Morhenn VB: Recombinant gamma interferon differentially regulates class II antigen expression and biosynthesis on cultured normal human keratinocytes. J Interferon Res 5:23–27, 1985
10. Nickoloff BJ, Basham TY, Merigan TC, Morhenn VB: Antiproliferative effects of recombinant alpha and gamma interferon on cultured human keratinocytes. Lab Invest 51:697–701, 1984
11. Nickoloff BJ, Basham TY, Merigan TC, Morhenn VB: Keratinocyte class II histocompatibility antigen expression. Br J Dermatol 112:373–374, 1985
12. Dorf ME: The Role of the Major Histocompatibility Complex in Immunobiology. New York, Garland Publishing, 1981
13. Pober JS, Collins T, Gimbrone MA, Cotran RS, Gitlin JD, Fiers W, Clayberger C, Krensky AM, Burakoff SJ, Reiss CS: Lymphocytes recognize human vascular endothelial and dermal fibroblast Ia antigens induced by recombinant immune interferon. Nature 305:726–729, 1983
14. Groenewegen G, Burman WA, Jeunhomme GMA, van der Linden CJ, Vegt PA, Kootstra G: In vitro stimulation of lymphocytes by vascular endothelial cells. Transplantation 37:206–210, 1984
15. Ashida ER, Johnson AR, Lipsky PE: Human endothelial cell-lymphocyte interaction. J Clin Invest 67:1490–1499, 1981
16. Hirschberg H, Evensen SA, Henriksen T, Thorsby E: The human mixed lymphocyte-endothelium culture interaction. Transplantation 19:495–504, 1975
17. Liu SC, Karasek M: Isolation and growth of adult human epidermal keratinocytes in cell culture. J Invest Dermatol 71:157–162, 1978
18. Morhenn VB, Nickoloff BJ, Mansbridge JN: Induction of the synthesis of triton-soluble proteins in human keratinocytes by gamma interferon. J Invest Dermatol 85(suppl):27s–29s, 1985
19. Snyder DS, Beller DI, Unanue ER: Prostaglandins modulate macrophage Ia expression. Nature 299:163–165, 1982
20. Morhenn VB, Engleman E, Farber EM: Significance of HLA antigens and the mixed lymphocyte reaction in psoriasis. Acta Derm Venereol [Suppl] (Stockh) 87:12–14, 1979
21. Geppert TD, Lipsky PE: Gamma interferon induced antigen presenting function by endothelial cells and fibroblasts. Clin Res 33:376A, 1985
22. Harris G, Olsen I: Cell division and DNA synthesis in cultures of stimulated lymphocytes. Immunology 31:195–204, 1976
23. Trinchieri G, Perussia B: Immune interferon: a pleiotropic lymphokine with multiple effects. Immunology Today 6:131–136, 1985
24. Berman B, Smith B: Amcinonide and gamma-interferon modulation of epidermal Langerhans cell DR expression. Clin Res 33:22A, 1985
25. Klaus GGB, Hawrylowicz CM: Cell-cycle control in lymphocyte stimulation. Immunology Today 5:15–19, 1984
26. Sontheimer RD: The mixed epidermal cell-lymphocyte reaction II. Epidermal Langerhans cells are responsible for the enhanced allogeneic lymphocyte-stimulating capacity of normal human epidermal cell suspensions. J Invest Dermatol 85(suppl):21s–25s, 1985
27. Umetsu DT, Katzen D, Jabara HH, Geha RS: Antigen presentation by human dermal fibroblasts: activation of resting T lymphocytes. J Immunol 136:440–445, 1986
28. Walker C, Kristensen F, Bettens F, Deweck AL: Lymphokine regulation of activated (G_1) lymphocytes. J Immunol 130:1770–1773, 1983
29. Stockman GD, Mumford DM: The effect of prostaglandin on the in-vitro blastogenic response of human peripheral blood lymphocytes. Exp Hematol 2:65–72, 1974
30. Goodwin JS, Bankhurst AD, Messner RP: Suppression of human T cell mitogenesis by prostaglandin. J Exp Med 146:1719–1734, 1977
31. Stobo JD, Kennedy MS, Goldyne ME: Prostaglandin E modulation

of the mitogenic response of human T cells. J Clin Invest 64:1188–1195, 1979

32. Denbow CJ, Conroy JM, Elgert KD: Macrophage derived prostaglandin E modulation of the mixed lymphocyte reaction: an anomaly of increased production and decreased T cell susceptibility during tumor growth. Cell Immunol 84:1–13, 1984
33. Yaron M, Yaron I, Wiletzki C, Zor U: Interrelationship between stimulation of prostaglandin E and hyaluronate production by polyIC and interferon in synovial fibroblast culture. Arthritis Rheum 21:694–698, 1978
34. Korn JH, Kalushka PV, LeRoy EC: Mononuclear cell modulation of connective tissue function. J Clin Invest 65:543–554, 1980
35. Tsuchida T, Iikima M, Fujiwara H, Pehamberger H, Shearer GM, Katz SI: Epidermal Langerhans cells can function as stimulatory cells but not as accessory cells in CTL induction. J Immunol 132:1163–1168, 1984
36. Nicolas JF, Kaiserlian D, Fanure J, Gigli I, Thivolet J: Immunological properties of an epidermal cell derived lymphocyte differentiating factor (abstr). J Invest Dermatol 84:303, 1985